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(54) Nucleotide Sequences for Bovine Sex Determination

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ABSTRACT OF THE DISCLOSURE

The invention relates to purified and isolated nucleotide sequences from the Y chromosome and autosomal chromosomes of bovines. Single strand DNA hybridization primers are provided which hybridize to the Y chromosome or autosomal sequences and which can be used to detect the presence of Y chromosome and autosomal DNA respectively. The nucleotide sequences may be used to determine the sex of bovines. Thus the invention also relates to methods of detecting the presence of Y chromosomal DNA and autosomal DNA respectively; and to methods of determining the sex of a bovine cell using the nucleotide sequences and hybridization primers of the invention.

BP File No. 6580-003/SMcD

Title: NUCLEOTIDE SEQUENCES FOR BOVINE SEX DETERMINATION

FIELD OF THE INVENTION

The invention relates to novel nucleotide sequences isolated from the Y chromosome and autosomal DNA of bovines, and fragments thereof; hybridization primers which are capable of hybridizing with the novel sequences; use of the sequences and hybridization primers to detect the presence of Y chromosomal DNA and autosomal DNA respectively; methods of detecting the presence of Y chromosomal and autosomal DNA using the sequences and hybridization primers of the invention; and methods of determining the sex of a bovine cell using the sequences and hybridization primers of the invention.

BACKGROUND OF THE INVENTION

Y specific probes constructed from DNA sequences specific to the bovine Y chromosome have been used to detect the sex of animal cells, for example from bovine preimplantation embryos (see, for example Ellis, U.S. Patent No. 4,769,319; Kwoh and Gingeras, PCT Application No. US90/03318; Herr et al, A. & N.Z. Soc. Study Cell Biol. Proc. 1989; Higashi et al, Jpn. J. Anim. Reprod. 37:115, 1991; and Miller and Koopman, Animal Genet. 21:77, 1990).

Several primers have been identified which have some degree of specificity for bovine Y chromosomes, and which can be used in a polymerase chain reaction (PCR) to provide an indication of the sex of bovine embryo cells (Kwoh and Gingeras, PCT Application No. US90/03318; Reed et al, PCT Application No.AU89/0029; Herr et al, A. & N.Z. Soc. Study Cell Biol. Proc. 1989; Bredbacka et al, Reprod. Dom. Anim. 26:75, 1991; Miller, Reprod. Dom. Anim. 26:58, 1991; Glasgow, New Scientist 9:31, 1989; Schwerin et al, Reprod. Dom. Anim. 26:70, 1991; and Higashi et al, Jpn. J. Anim. Reprod. 37:115, 1991). Bovine sexing by PCR depends on the selective amplification of a DNA sequence only

found in male embryos i.e. on the Y chromosome. Thus male cells are recognised on the basis of amplification of the male specific DNA sequence, and female cells are recognised by the absence of an amplification reaction.

Accurate sex determination by PCR amplification depends on the specificity of the Y-specific hybridization primers and on a reliable control reaction which allows a true negative result (female) to be distinguished from cases where the PCR reaction is inadequate for some extraneous reason, such as lack of DNA in the sample, faulty reagents or inappropriate reaction conditions. The simultaneous amplification of an autosomal sequence has been used as a control for amplification (Kwoh and Gingeras, PCT Application No. US90/03318; Reed et al, PCT Application No.AU89/0029; Herr et al, A. & N.Z. Soc. Study Cell Biol. Proc. 1989; Bredbacka et al, Reprod. Dom. Anim. 26:75, 1991; and Schwerin et al, Reprod. Dom. Anim. 26:70, 1991).

In order to be commercially viable and useful, it is desirable that a method of sex determination be as close to 100% accurate as possible. The accuracy of PCR sexing of embryonic cells depends upon the interplay of a number of factors, including the sensitivity and specificity of the assay. These factors depend to a large extent on the stringency of the Y-specific hybridization primers for Y-specific DNA and the complete lack of a reaction with any non Y-specific DNA. It is also advantageous for the autosomal control reaction to be reliable and easily distinguishable from a positive male reaction.

SUMMARY OF THE INVENTION

The present inventors have identified novel nucleotide sequences highly specific for the Y chromosome and autosomal DNA of bovines which permit the accurate determination of the sex of bovine cells, particularly from embryos or fetuses.

The present invention provides a purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5.

The invention relates to a purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.

The invention also relates to a purified and isolated nucleotide sequence having substantial homology to the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.

The invention further provides fragments of the above sequences, including any contiguous portion of the above sequences consisting of 15 or more nucleotides, preferably comprising the sequence 5-1 or 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 or K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5.

The invention further provides a purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a sequence having substantial homology thereto which is capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA. The invention also relates to fragments, or any contiguous portion consisting of 15 or more nucleotides, of the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a

sequence having substantial homology thereto which is capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA.

The invention further provides fragments of the above-mentioned nucleotide sequences, preferably comprising the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto.

The invention also relates to a method of detecting the presence of autosomal DNA or Y chromosomal in a tissue or cell sample, preferably a bovine tissue or cell sample, most preferably from a member of the genus Bos, using fragments of the above-mentioned nucleotide sequences.

The invention also relates to a method of determining the sex of a bovine cell by isolating a DNA sample from the cell; treating the sample with one or more pairs of Y-specific hybridization primers, which are capable of amplifying a nucleotide sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, or a sequence having substantial homology thereto, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product, whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

In a preferred embodiment, the invention relates to a method of determining the sex of a bovine cell, preferably a bovine cell from an embryo or amniotic fluid using fragments of the Y specific nucleotide sequences of the invention as hybridization primers, which method comprises obtaining a DNA sample from the cell; treating the sample with one or more pairs of hybridization primers selected from the group consisting of the sequences 5-1 and 6-1

shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5, under hybridization conditions in the polymerase chain reaction to produce amplified product, and; detecting the amplified product, whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

In a particularly preferred embodiment, the DNA sample is simultaneously treated, as a control, with one or more pairs of hybridization primers comprising the fragments JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions are confirmed by the presence of autosomal amplification product.

In a further preferred embodiment the invention provides a kit for determining the sex of a bovine cell comprising a pair of hybridization primers which are complementary to a fragment of the nucleotide sequence Y1 shown in SEQ ID NO:3 and Figure 3, Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, and which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA under hybridization conditions in the polymerase chain reaction (PCR) to produce a Y chromosomal amplification product; the reagents required for the PCR reaction and; means for detecting the amplification product.

In a particularly preferred embodiment, the invention provides a kit for carrying out the method of sexing

bovine cells, comprising a pair of hybridization primers, preferably 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and K2 shown SEQ ID NO:10 and SEQ ID NO:11 respectively and in Figure 5 or sequences having substantial homology thereto, which under hybridization conditions in the polymerase chain reaction (PCR) amplify a Y chromosomal product, means for detecting the product and, the reagents required for the PCR reaction. Preferably, the kit is further composed of a pair of hybridization primers, most preferably the further pair of hybridization primers have the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which amplify an autosomal sequence.

The invention also relates to a method of detecting the presence of Y chromosomal DNA in a tissue or cell sample, preferably a tissue or cell sample from a bovine, most preferably from a member of the genus Bos, comprising selecting one or more fragments of the invention which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA, contacting the sample under hybridization conditions with one or more of the fragments which are labelled with a detectable marker and determining the degree of hybridization between the DNA of the sample and the fragment.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the hybridization primers used to amplify the 157 bp segment of bovine male DNA, BOV97M;

Figure 2 shows the restriction map of the Eco RI fragment of the 20 kb segment of male bovine DNA;

Figure 3 shows the DNA sequence of Y1;

Figure 4 shows the DNA sequence of Y2;
Figure 5 shows the DNA sequence of Y3;
Figure 6 shows the DNA sequence of AUTO1;
Figure 7 shows the DNA sequence of AUTO2; and
Figure 8 is a diagrammatic illustration of a gel showing the results of PCR amplification for the determination of the sex of bovine cells.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention relates to a purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, or a sequence having substantial homology thereto, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA. The invention provides fragments of the above sequences, preferably comprising the sequence 5-1 or 6-1. shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 or K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5.

It will be appreciated that the invention includes nucleotide sequences which have substantial sequence homology with the above-mentioned nucleotide sequences shown in the Figures. The term "sequences having substantial sequence homology" used herein means those nucleotide sequences which have slight or inconsequential sequence variations from the sequences disclosed in SEQ ID NOS: 1 to 17 and in Figures 1, and 3-7, i.e. the homologous sequences function in substantially the same manner. It is understood that nucleic acid molecules can tolerate a certain number of mismatched base pairs and still form duplexes. Accordingly a degree of mismatch can be tolerated in a hybridization reaction. The variations may be attributable to local mutations or structural modifications.

It will also be appreciated that a double stranded nucleotide sequence comprising a DNA segment of the invention or an oligonucleotide fragment thereof, hydrogen bonded to a complementary nucleotide base sequence, an RNA made by transcription of this doubled stranded nucleotide sequence, and an antisense strand of a DNA segment of the invention, an oligonucleotide fragment encoded by the DNA segment, or a complementary sequence of a DNA sequence of the invention or fragment thereof, are contemplated within the scope of the invention.

The nucleotide sequences of the invention can be prepared, for example, by the method generally described below and particularly described in the examples.

Sequences from the Y chromosome of bovines may be identified by screening a bovine male genomic library with a known sequence from the Y chromosome, for example BOV97M described by Miller and Koopman (Animal Genetics, 21:77, 1990). A bovine male genomic library may be constructed in the vector lambda FIX II (Stratagene) using a preparation of total male bovine DNA. The term "male bovine DNA" refers to DNA extracted from male bovine tissue, preferably the liver.

The bovine male genomic library may be screened with the Y chromosome specific DNA probe BOV97M. This probe may be obtained from male bovine DNA, for example by PCR amplification. Preferably, the BOV97M sequence may be amplified by the pair of primers shown in SEQ ID NOS:1 and 2 and in Figure 1, which are complementary to the ends of the 157 bp segment BOV97M.

After PCR amplification, the BOV97M fragment may be ligated into a cloning vector, preferably pT218U (Pharmacia), and transformed into a eukaryotic or prokaryotic cell, preferably a bacterium, most preferably E. coli. The BOV97M fragment may be prepared in quantity and radiolabelled by nick translation. It will be

appreciated that the term "nick translation" refers to the incorporation of deoxynucleoside triphosphates (dNTPs), preferably radiolabelled, at nicks introduced into a nucleotide sequence by limited treatment with DNase I. It will also be appreciated that other techniques for labelling nucleotides are known in the art.

The radiolabelled BOV97M probes may be used to screen a bovine male genomic library and to identify sequences of Y chromosomal DNA, preferably a 20 kb sequence of Y chromosome DNA.

The nucleotide sequences of the invention may also be synthetically synthesized by methods known in the art. For example, they may be synthesized by the phosphoramidite procedure on an automated synthesizer followed by purification by thin layer chromatography as described generally in T.K. Archer et al (1985) J. Biol. Chem. 260:1676-1681. Sequences may also be amplified using the polymerase chain reaction (PCR) which is discussed in more detail below.

A number of unique restriction sequences for restriction enzymes are incorporated in the 20 kb sequence, identified in Figure 2, and provide access to novel Y chromosomal nucleotide sequences. It is possible to make use of the recognition sites for restriction enzymes, shown in Figure 2, to prepare partial sequences of the 20 kb sequence. In particular, the 5 kb Eco R1 partial sequence may be subcloned and several nucleotide sequences within the 5 kb partial sequence may be subcloned and sequenced. For example, the nucleotide sequences Y1, Y2 and Y3 are shown in SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9 and Figures 3, 4 and 5 respectively. DNA fragments unique to Y1, Y2 and Y3 can also be constructed by chemical synthesis and enzymatic ligation reactions carried out in a manner known per se.

As hereinbefore mentioned, the invention relates to a

purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a nucleotide sequence having substantial homology thereto which is capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA.

The invention further provides fragments of the above-mentioned nucleotide sequences, designated AUTO 1 and AUTO 2, preferably comprising the fragments JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto.

It is understood that the term "autosomal" refers to genetic material contained in any chromosome other than the Y and X sex chromosomes. The term "sequences having substantial homology thereto" has been previously discussed.

The nucleotide sequences of the invention hybridizing to a significantly greater extent with autosomal DNA may be prepared from bovine genomic DNA, for example by the following method.

Genomic male bovine DNA, may be ligated into a vector, preferably the commercially available vector, PTZ18U and transformed into a prokaryotic or eukaryotic cell, preferably a bacterium, most preferably E. coli. The DNA from several transformants may be prepared in quantity and labelled by nick translation. Labelled probes may be selected which hybridize to autosomal DNA present in male and female bovine genomic DNA. Preferably autosomal probes are selected which hybridize to autosomal DNA with approximately the same efficiency as the Y specific probes hybridize to male DNA. The most preferred autosomal nucleotide sequences of the invention AUTO1 and AUTO2 are shown in SEQ ID NO:12 and SEQ ID NO:15 and Figures 6 and

7 respectively.

The nucleotide sequences, sequences having substantial homology thereto and DNA fragments having sequences unique to AUTO1 and AUTO2 can also be constructed by chemical synthesis and enzymatic ligation reactions carried out using methods known in the art as discussed above.

The nucleotide sequences of the invention or fragments of the nucleotide sequences, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences in biological materials such as DNA samples, tissues or body fluids. A nucleotide probe may be labelled with a radioactive label which provides for an adequate signal and has sufficient half-life such as ^{32}P, ^{3}H, ^{14}C or the like. Other labels which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization.

The sequences of the present invention permit the identification and isolation or synthesis of fragments of the nucleotide sequences of the invention which may be used as primers to amplify a corresponding selected nucleotide sequence having a sequence complementary to the primer, for example in the PCR reaction. The present invention also provides pairs of primers which are selected such that they amplify nucleotide sequences of the invention, preferably under hybridizing conditions in the PCR reaction. Primers which may be used to amplify nucleotide sequences which are specific to bovine Y chromosomal DNA include: 5-1 and 6-1 for the sequence Y1, shown in SEQ ID NO:3 and Figure 3; K3 and K4 for the sequence Y2 shown in SEQ ID NO:6 and Figure 4; K1 and K2 for the sequence Y3, shown in SEQ ID NO:9 and Figure 5.

Primers which may be used to amplify sequences which are specific to autosomal DNA include: JC4 and JC7 for the sequence AUTO1, shown in SEQ ID NO:12 and Figure 6; and JK1 and JK2 for the sequence AUTO2, shown in SEQ ID NO:15 and Figure 7.

As hereinbefore mentioned, in a preferred embodiment, the invention relates to a method of determining the sex of a bovine cell, preferably from an embryo, which method comprises isolating a DNA sample from the cell; treating the sample with at least one pair of hybridization primers which are capable of amplifying the Y-specific sequences of the invention, preferably the pair of hybridization primers 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and in Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product, whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

In a particularly preferred embodiment the DNA sample is simultaneously treated, as a control, with at least one of the following pairs of hybridization primers: JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or primers having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions are confirmed by the presence of autosomal amplification product.

It will be appreciated that the term "hybridization conditions" refers to reaction conditions which permit hybridization and amplification reactions to proceed in

the presence of the DNA sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art.

The term "polymerase chain reaction (PCR)" used herein refers to the process for amplifying a target nucleotide sequence as generally described in Innis et al (ed) PCR Protocols, Academic Press, 1990 and U.S. Patent 4,800,159 to Mullis et al. Amplification of the target nucleotide sequence may be accomplished by means of a pair of hybridization primers which flank the nucleotide sequence to be amplified. The primers hybridize to opposite strands of the target nucleotide sequence and DNA synthesis proceeds across the region between the hybridization primers, thereby doubling the amount of that DNA segment. Repeated cycles of denaturation, priming and extension permit rapid exponential amplification of the target nucleotide sequence. The amplified product may then be readily detected by standard techniques, for example by gel electrophoresis or autoradiography, preferably gel electrophoresis.

The term "hybridization primer(s)" refers to an oligonucleotide isolated and purified from a cell or produced by synthesis, which is capable of hybridizing to a complementary nucleotide sequence and acting as a point of initiation of synthesis when placed under conditions appropriate for the PCR reaction, in which synthesis of a primer extension product which is complementary to a nucleotide sequence is initiated. The hybridization primer generally contains 15 to 30 nucleotides and is preferably single stranded. The primers may be synthesized using methods known in the art.

The term "pair(s) of hybridization primers" refers to two hybridization primers which flank the nucleic acid sequence to be amplified in the PCR reaction. The two hybridization primers are selected so that they

sufficiently hybridize to different strands of the nucleotide sequence to be amplified at relative positions along the sequence such that an extension product synthesized from one primer when it is separated from its complementary strand will serve as a template for the synthesis of an extension product for the other primer. Preferred pairs of hybridization primers of the invention are 5-1 and 6-1, K3 and K4 and K1 and K2. Preferred pairs of hybridization primers which may be used to selectively amplify autosomal DNA are JC4 and JC7, and JK1 and JK2. The DNA sequences of the pairs of hybridization primers are shown in Figures 3, 4, 5, 6 and 7 respectively.

It will be appreciated that a hybridization primer may be modified provided a sufficient amount of the hybridization primer contains a sequence which is complementary to the strand to be amplified. For example, it may be modified to assist in isolating amplified product by introducing a restriction site in the primer.

The DNA sample may be incubated with deoxynucleoside triphosphates (dNTPs), preferably at a concentration of 0.05 to 0.5mM, most preferably 0.2 mM, as well as a pair of bovine Y chromosomal hybridization primers and a pair of control autosomal hybridization primers. To this may be added Taq polymerase, preferably 1 to 5 units, most preferably 2.5 units and the reaction may be carried out preferably for 20 to 70 cycles, most preferably for 45 cycles. In a preferred embodiment, the annealing temperature may be 62°C and the elongation temperature may be 72°C during each cycle. The reactions may be terminated, for example, by the addition of ethanol to precipitate the DNA. The resulting DNA may be analyzed to identify autosomal and Y chromosomal sequences by standard techniques, preferably gel electrophoresis.

The Y chromosomal hybridization primers may be used to accurately determine the sex of animals, preferably bovines, most preferably of the genus Bos, in DNA samples

extracted from a few cells, preferably 3-5 cells. The cells may be obtained from a pre-implantation bovine embryo, preferably a seven day old embryo, or from cells obtained from a sample of amniotic fluid, for example by the following method.

The DNA from several cells of a bovine embryo may be extracted using techniques known in the art and described, for example in Innis et al (ed.), PCR Protocols, Academic Press, 1990, and treated with the Y chromosomal hybridization primers of the invention, preferably with one pair of hybridization primers under hybridization conditions in the PCR reaction, to selectively produce an amplified product i.e. specific sequences on the Y chromosome. Detection of the amplified product indicates a male embryo and the lack of an amplification product indicates a female embryo.

The amplified products may be readily isolated and distinguished by techniques known in the art. Preferably, the amplified products may be distinguished based on their respective sizes using techniques known in the art. For example, after amplification, the DNA sample can be separated on an agarose gel and visualized, after staining with ethidium bromide, under ultra violet (UV) light.

In a preferred embodiment of the invention, the DNA sample from the embryo to be sexed may be simultaneously treated with a pair of Y chromosomal hybridization primers and a pair of autosomal hybridization primers.

In a particularly preferred embodiment the DNA sample may be treated with the following pairs of hybridization primers: JK1 and JK2, which amplify a 113 bp autosomal sequence and K1 and K2 which amplify a 199 bp Y chromosome sequence; or JC4 and JC7 which amplify a 174 bp autosomal sequence and J5-1 and J6-1 which amplify a 700 bp Y chromosome sequence. It is advantageous to use the above-noted pairs of hybridization primers in the particularly

preferred embodiment of the invention as the Y-specific and autosomal pairs of hybridization primers amplify respective products which have significant size differences and which can be readily and clearly separated by size by techniques such as gel electrophoresis. Thus, this particularly preferred embodiment provides for a rapid and clear determination of genetic sex, as illustrated in Figure 8.

The amplification products may be used to determine the sex of the embryo, as illustrated in Figure 8. Amplification of the autosomal sequence acts as a control to confirm the presence of DNA and hybridization primers under hybridization conditions. Amplification of the Y chromosome sequence designates the embryo as male. Lack of amplification of the Y chromosome sequence, combined with amplification of the autosomal sequence designates the embryo as female.

As hereinbefore mentioned, the invention also provides a kit for carrying out the method of sexing bovine cells, comprising a pair of hybridization primers, preferably having the sequences 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO: 11 respectively and Figure 5 or sequences having substantial homology thereto, which under hybridization conditions in the polymerase chain reaction (PCR) amplify a Y chromosomal product, means for detecting the amplified product as discussed above and, the reagents required for the PCR reaction. Preferably, the kit is further composed of a pair of hybridization primers, most preferably the further pair of hybridization primers have the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which amplify an autosomal sequence.

It is an advantage of the nucleotide sequences of the present invention that they permit accurate sexing of embryos with a success rate at or close to 100%.

The nucleotide sequences of the invention and fragments thereof are shown in Figures 1 and 3-7 and are also shown in the appended Sequence Listing as follows:

SEQ ID NO:1 shows the first probe sequence in Figure 1
SEQ ID NO:2 shows the second probe sequence in Figure 1
SEQ ID NO:3 shows Y1 in Figure 3
SEQ ID NO:4 shows 5-1 in Figure 3
SEQ ID NO:5 shows 6-1 in Figure 3
SEQ ID NO:6 shows Y2 in Figure 4
SEQ ID NO:7 shows K3 in Figure 4
SEQ ID NO:8 shows K4 in Figure 4
SEQ ID NO:9 shows Y3 in Figure 5
SEQ ID NO:10 shows K1 in Figure 5
SEQ ID NO:11 shows K2 in Figure 5
SEQ ID NO:12 shows AUTO1 in Figure 6
SEQ ID NO:13 shows JC4 in Figure 6
SEQ ID NO:14 shows JC7 in Figure 6
SEQ ID NO:15 shows AUTO2 in Figure 7
SEQ ID NO:16 shows JK1 in Figure 7
SEQ ID NO:17 shows JK2 in Figure 7

The following non-limiting examples are illustrative of the present invention:

Example 1

Preparation of Bovine Genomic DNA

DNA prepared according to this Example from male and female bovine tissue is also referred to in the specification as "male bovine DNA" and "female bovine DNA" respectively.

Male and female bovine liver biopsies were obtained from Holsteins slaughtered at the University of Guelph abattoir. The liver biopsies from each sex were processed

separately by identical procedures to obtain male and female bovine genomic DNA. Bovine genomic DNA was extracted from male and female liver biopsies by the following procedure to produce male bovine DNA and female bovine DNA.

A 0.25 cm^3 tissue fragment of the liver biopsy was added to 0.7 ml of 50 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS. Proteinase K was added to a concentration of 0.5 mg/ml, and the sample incubated at 55°C for 16 hours. Following incubation, the DNA was extracted with phenol and chloroform generally following the procedure outlined in Sambrook et al, Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press. Sodium acetate (pH 6.0) was added to a concentration of 0.3M, and the DNA was collected by precipitation with an equal volume of ethanol at room temperature. The ethanol precipitated DNA was pelleted by centrifugation at 13,000 X g, and the resulting DNA pellets were dissolved in 100 µl of 10 mM Tris, pH 8.0, 1 mM EDTA.

Example 2

Construction of Bovine Male Genomic Library and Isolation of Y Chromosome DNA.

Total male bovine DNA, prepared as described in Example I, was used to construct a bovine male genomic library. A library of bovine male DNA was constructed in the vector Lambda FIX II (Stratagene), generally following the manufacturer's instructions. The genomic DNA was partially digested with Sau 3A, generating fragments in the range of 10-50 kb. The fragments were ligated to the lambda arms of the phage vector and the recombinant phage were packaged in vitro following the instructions provided by the manufacturer of the vector.

The resulting library was plated and screened with a DNA probe specific for a 157 bp segment of Y chromosome DNA, (BOV97M). The Y chromosome DNA probe BOV97M, described by Miller and Koopman (Animal Genetics, 21:77, 1990) was

obtained by PCR amplification from bovine male genomic DNA. The PCR amplification was carried out following the method of Innis et al (PCR Protocols, Academic Press, 1990). The two hybridization primers used in the PCR amplification were prepared using the standard cyanoethyl phosphoramidite synthesis method, and are shown in SEQ ID NOS: 1 and 2 and Figure 1. The hybridization primers are complementary to the ends of the 157 bp segment, BOV97M.

After PCR amplification, the DNA band corresponding to BOV97M was purified on a low melting point agarose gel, and ligated into the Sma I site in the polylinker of the cloning vector pTZ18U (Pharmacia). The plasmid was transformed into E. coli strain DH5α. The identity of the insert was confirmed by DNA sequence analysis, and the BOV97M fragment was prepared in quantity and radiolabelled by nick translation, following the methods of Sambrook et al supra.

The radiolabelled BOV97M fragment was used to screen the bovine male genomic library for positive clones. The labelled fragment was hybridized to a plaque lift of the bovine male genomic library using standard procedures (Sambrook et al, Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989). One positive clone was identified. The phage DNA within the positive clone was analyzed and it was found to contain a 20 kilobase pair of Y chromosome DNA, within which was present the 157 bp BOV97M segment. A restriction map of the 20 kb DNA sequence is shown in Figure 2.

A 5 kb Eco RI fragment from the 20 kb insert was subcloned in the Eco RI site of pTZ12U, and several fragments within it were subcloned and sequenced. For example, several Bgl II fragments were identified within the 5 kb Eco RI fragment and are shown in Figure 2. The Bgl II fragments Y1, Y2 and Y3, shown in Figure 2, were again subcloned, into the Bam HI site of PTZ18U, and sequenced. The

nucleotide sequences of the fragments Y1, Y2 and Y3 are shown in SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9 and Figures 3, 4, and 5, respectively.

Example 3

Preparation of Hybridization Primers Specific for Male Bovine DNA

Using the sequence data obtained for the Bgl II fragments, Y1, Y2 and Y3, oligonucleotide hybridization primers for use in PCR amplification of the fragments were chemically synthesized by the standard dideoxy sequencing method as described by Sambrook et al supra. The hybridization primers are complementary to sequences at, or near to, the ends of the fragments. The hybridization primers for the Y1 fragment, designated 5-1 and 6-1 are shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and are shown underlined on Figure 3; the hybridization primers for the Y2 fragment, designated K3 and K4 are shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and are shown underlined on Figure 4; and the hybridization primers for the Y3 fragment, designated K1 and K2, are shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and are shown underlined on Figure 5.

Example 4

Preparation of Hybridization Primers for Autosomal Bovine DNA

Male bovine genomic DNA that had been digested with Sau 3A was ligated into the Bam HI site of PTZ18U and transformed into E. coli. Plasmid DNA was prepared from several transformants and labelled by nick translation. These probes were hybridized to male or female genomic DNA that had been spotted onto nitrocellulose. All of the probes tested in this way hybridized to both male and female genomic DNA, indicating that they did not contain Y specific DNA and were from autosomal sequences. This was not surprising considering that the great majority of genomic DNA in males is also present in females.

The cloned segments in two of these plasmids, AUTO1 and AUTO2, were sequenced and oligonucleotide hybridization primers synthesized that would amplify portions of them in a PCR reaction. The hybridization primers for AUTO1, JC4 and JC7 are shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and are shown underlined on Figure 6; and the hybridization primers for AUTO2, JK1 and JK2 are shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and are shown underlined on Figure 7.

Example 5

Sex Determination of Bovine Embryos

Three to five cells from a bovine embryo to be sexed were removed by micromanipulation from seven day old embryos, and placed into 20 µl of sterile H_2O. The cell samples were either used directly at this stage or were frozen for subsequent analysis. The embryos from which the samples were removed were labelled and maintained in vitro pending the outcome of the sex determination prior to implantation into recipient cows.

The sex of the embryo from which the cells were removed was determined by PCR amplification using a combination of bovine male specific hybridization primers and bovine autosomal hybridization primers. Two combinations were most commonly used. One combination was the male specific hybridization primers K1 and K2 with the autosomal hybridization primers JK1 and JK2; and the other was the male specific hybridization primers J5-1 and J6-1 with the autosomal hybridization primers JC4 and JC7.

For analysis, the sample volume was made up to 50 µl using PCR buffer (final concentration 50 mM KCl, 10 mM tris, pH 8.4, 1.5 mM $MgCl_2$) SDS was added to a final concentration of 2.0 µM, and proteinase K to a concentration of 50 µg/ml. The sample was then incubated for one hour at 55°C, and the proteinase K was then inactivated by heating to 95°C for 10 minutes.

Following incubation, deoxynucleoside triphosphates (dNTPs) were added (0.2 mM final concentration), as well as a set of bovine male specific hybridization primers and a set of control autosomal hybridization primers (0.15 µg of male hybridization primers and 0.10 µg of autosomal hybridization primers). The final volume was 100 µl in PCR buffer. To this was added 2.5 units of Taq polymerase, and the reactions were then carried out for 45 cycles using an annealing temperature of 62°C and an elongation temperature of 72°C during each cycle. The reactions were terminated by the addition of two volumes of ethanol to precipitate the DNA.

The DNA was pelleted by centrifugation. The DNA pellets were resuspended in 15 µl of gel loading buffer (3% Ficoll, 0.03% xylene cyanol), and run on a 1.5% agarose gel. The gel was then stained in ethidium bromide and the DNA visualized on a UV light transilluminator. The sex of the embryo was determined as shown in Figure 8. The embryo was determined to be female based upon the presence of one amplified band on the gel, representing the autosomal sequence. The embryo was determined to be male based upon the presence of two amplified bands on the gel, representing the male specific sequence and the autosomal sequence. Polaroid photographs of the gel were taken to provide a permanent record of the results.

To confirm the sex of the embryo tested by the above PCR amplification reaction the embryos were transferred to recipient cows and carried to term.

A total of over 100 embryos have been sexed by means of the above PCR reaction and 89 sexed embryos have been transferred to recipient cows. Sixteen live calves have been produced and the sex of the embryos as determined by the PCR reaction of the present invention was confirmed in all cases by the sex of calf produced.

The present invention has been described in detail and

with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

Forming part of the present disclosure are the appended sequence listings.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Wildeman, Alan G.
Kelly, John J.

(ii) TITLE OF INVENTION: Nucleotide sequences for bovine sex determination

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Linda M. Kurdydyk, Bereskin & Parr
(B) STREET: Box 401, 40 King Street West
(C) CITY: Toronto
(D) STATE: Ontario
(E) COUNTRY: Canada
(F) ZIP: M5H 3Y2

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kurdydyk, Linda M.
(B) REGISTRATION NUMBER: 34,971
(C) REFERENCE/DOCKET NUMBER: 6580-003

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (416) 361-7311
(B) TELEFAX: (416) 361-1398
(C) TELEX: 06-23115

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCACTATA CATACACCAC TCTCAT 26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGATAAAAAG GCTATGCTAC ACAAAT 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 745 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II (Stratagene)
(B) CLONE: Y1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCTTGGGA GCAAGCAAAG TGTTTGGCCA CCTGAATAAT GTTGCCTAAA ATTTTATTTT 60

GACATATCCA CATACATACC CATGTGTGTA TGAGTTTGGG TATAAAAAAA TTACATTGAC 120

TTTTAATTCC CATTAGGTAT AACAACATGG AAAAATTAAA ACCCAAATGC AGAATGACTC 180

AAGACTCTAC ACAGTTATTG AATATATGTA AGTGGAAATA CTTCAAAATA TGTTTTATTG 240

GCTACATCTA ACATATAAGG ACTCTTTTAT ACCACTTCCC CTCTCTCCTT TTAATAAATA 300

CCTACCAATT TTCAAAGTTT GCCCAGGTTT ATGAAATACT CAAGGAATAA TAGGAAAAAT 360

ATATATATAT ATATATTATA TATATATATA GGAGAGGTAG GAACCAATCA TTACTCTGCT 420

GGCCAATCCA TGGATGGAGG AGCCTGGTAG GCTGCAGTCC ATGGTGTCAC AAAATAGATC 480

GGTACAACTG AGTGACTTCC CTTTCACTTT TCACTTTCAT GCATTGGAGA AGGACATAGC 540

AACCCACTCG AATGTTCTTG CCTGGAGAAT CCTGGGACAG ATGAGCCTGG TGGGCTGCCG 600

TCTGTGGGGT CACACAGAGT TGGACACGTC TGAAATGACA GCAGATATAC CTATTTATAT 660

GCATATTTAT GTATATAGAT ATACAAATAT CTATATAAAA TAGATATGTT TCCATTGCAG 720

AAAATGTTGA TACCAACCTT AGATC 745

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos Taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACCTGAATA ATGTTGCCTA AAATT 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTCTGCAAT GGAAACATAT CTATT 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 257 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTCAGAA TTATTTCCAA CACCATGCTC CAGGTCAACA GGAAATAGCC AAAGTTGTTG 60

CTGACACAAA CAGTTCAAGA GCTGGGAAGC CTGTAGACCT CCTCATTTTC CAAGAGTAAT 120

TTAATCAGAG AAGCTAGAAA TCCAGAAATT AAGGAAAATA ATCAAGCAAC AGAAGATAGT 180

AATGATACTT TCACTTTTAA CTTTCATGCA TTGGAGAAGG AAATGGCAAC CCATTCCAGT 240

GTTCTTCCCT GGAGAAT 257

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCTCAGAA TTATTTCCAA CACCAT 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromsome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATTCTCCAGG GAAGAACACT GGAAT 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 199 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus

(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y3

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromsome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATAGATCTCA AAACTGAGTC CCTAGTAAGT GTATAATCAT ACAAAGAAGA TCTAACGGAG 60

GTTTCTGAAC CAGTTGATTC ATCCTTAGGA GTCCCATCTT GAATTTCTGA AGAAATATGT 120

GCCATCTCAA AGAAAGTCTT TCTTCAGATA TTTTCTTATT TGTCTGATTT ACACACTGCA 180

ACTTCAAATG CTGCTTGGC 199

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y3

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromsome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAGATCTCA AAACTGAGTC CCTAGT 26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: Y3

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Y Chromsome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCAAGCAGC ATTTGAAGTT GCAGT 25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 174 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: AUTO1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCAGACTTC TATAGTTGAA ACCTGTGCTG GATTCTGAGA GCAAGAAACC TCCACCAGCC 60

TGATAAAACT GCATGGGAGA GTTGGTTCCA GACTGAACAA TGTGAGCATG GAAAAGCCAT 120

TCTTCTAATT AGATTGGCAG ACAGGAACAA CCTCTGCTAT GAGGTGAAAT ACAC 174

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: AUTO1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACCAGACTTC TATAGTTGAA ACCTGT 26

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: AUTO1

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGTATTTCA CCTCATAGCA GAGGTT 26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library lambda FIX II
(B) CLONE: AUTO2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCGAACTG ATGGTCCCTA TATTGCCAGG TGGATTCTTC ACTACTGGAC CACCAGGGAA 60

GTCCCTGATG TGTTTTTTTG TTTTTTTTTT TTTTAAAGTG AAGGATAATG CAGCTAAAAT 120

CAGCAGATC 129

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: AUTO2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAACTGATGG TCCCTATATT GCCAG 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bos taurus
(B) STRAIN: Holstein
(F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: Bovine male DNA library in lambda FIX II
(B) CLONE: AUTO2

(viii) POSITION IN GENOME:
(A) CHROMOSOME/SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTAGCTGCAT TATCCTTCAC TTTAA 25

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3; Y2 shown in SEQ ID NO:6; or Y3 shown in SEQ ID NO:9, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.

2. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y1 shown in SEQ ID NO:3.

3. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y2 shown in SEQ ID NO:6.

4. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y3 shown in SEQ ID NO:9.

5. A nucleotide sequence having substantial homology to the nucleotide sequence as claimed in claim 1.

6. A fragment of the nucleotide sequence as claimed in claim 1 or any contiguous portion thereof consisting of 15 or more nucleotides.

7. A fragment as claimed in claim 6 comprising the sequence 5-1 or 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5; K3 or K4 shown in SEQ ID NO:7 and SEQ ID NO:8; or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11 or a sequence having substantial homology thereto.

9. A purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12, or AUTO 2 shown in SEQ ID NO:15, which is capable of hybridizing to a significantly greater extent with autosomal chromosomal DNA than with Y chromosomal DNA.

10. A fragment of the nucleotide sequence claimed in claim 9 or any contiguous portion thereof consisting of 15 or more nucleotides.

11. A fragment as claimed in claim 10 comprising the sequence JC4 or JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 or JK1 or JK2 shown in SEQ ID NO:16 and SEQ ID NO:17, or a sequence having substantial homology thereto.

12. A method of determining the sex of a bovine cell, which method comprises isolating a DNA sample from the cell; treating the sample with one or more pairs of hybridization primers, which are capable of amplifying a sequence as claimed in claim 1 or 5, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product, whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

13. A method as claimed in claim 12 wherein the pairs of hybridization primers comprise 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11.

14. A method as claimed in claim 13 whereby as a control the DNA sample is simultaneously treated with one or more pairs of hybridization primers comprising JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17, or sequences having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions are confirmed by the presence of autosomal amplification product.

15. A method as claimed in claim 12 wherein the bovine

cell is from an embryo.

16. A kit for determining the sex of a bovine cell comprising a pair of hybridization primers which are ccmplementary to a fragment of the nucleotide sequence Y1 shown in SEQ ID NO:3, Y2 shown in SEQ ID NO:6; or Y3 shown in SEQ ID NO:9, and which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA under hybridization conditions in the polymerase chain reaction (PCR) to produce a Y chromosomal amplification product; the reagents required for the PCR reaction and; means for detecting the amplification product.

17. A kit as claimed in claim 16 which additionally comprises a pair of hybridization primers which are complementary to a fragment of the nucleotide sequence, AUTO 1 shown in SEQ ID NO:12, or AUTO 2 shown in SEQ ID NO:15, and which are capable of hybridizing to a significantly greater extent with autosomal chromosomal DNA than with Y chromosomal DNA under hybridization conditions in the polymerase chain reaction (PCR) to produce an autosomal amplification product.

18. A kit as claimed in claim 16 for use in determining the sex of bovine embryo cells.

19. A method of detecting the presence of Y chromosomal DNA in a tissue or cell sample comprising selecting a fragment as claimed in claim 6 labelled with a detectable marker, contacting the sample under hybridization conditions with the fragment and determining the degree of hybridization between the DNA of the sample and the fragment.

FIGURE 1 2113957

5'-GATCACTATACATACACCACTCTCAT-3'

5'-TGATAAAAAGGCTATGCTACACAAAT-3'

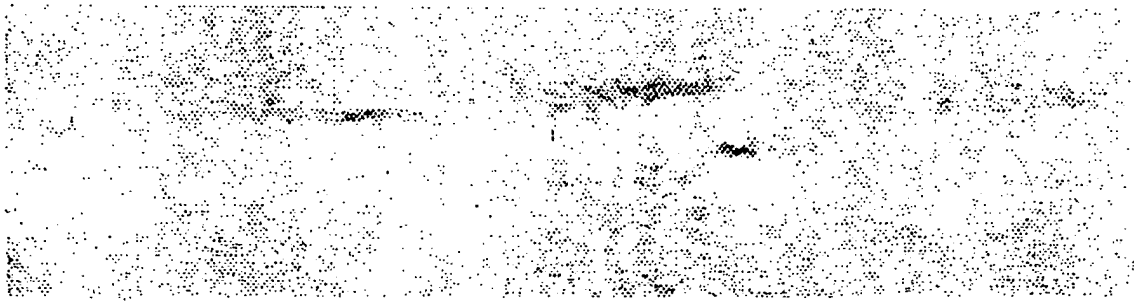

FIGURE 2

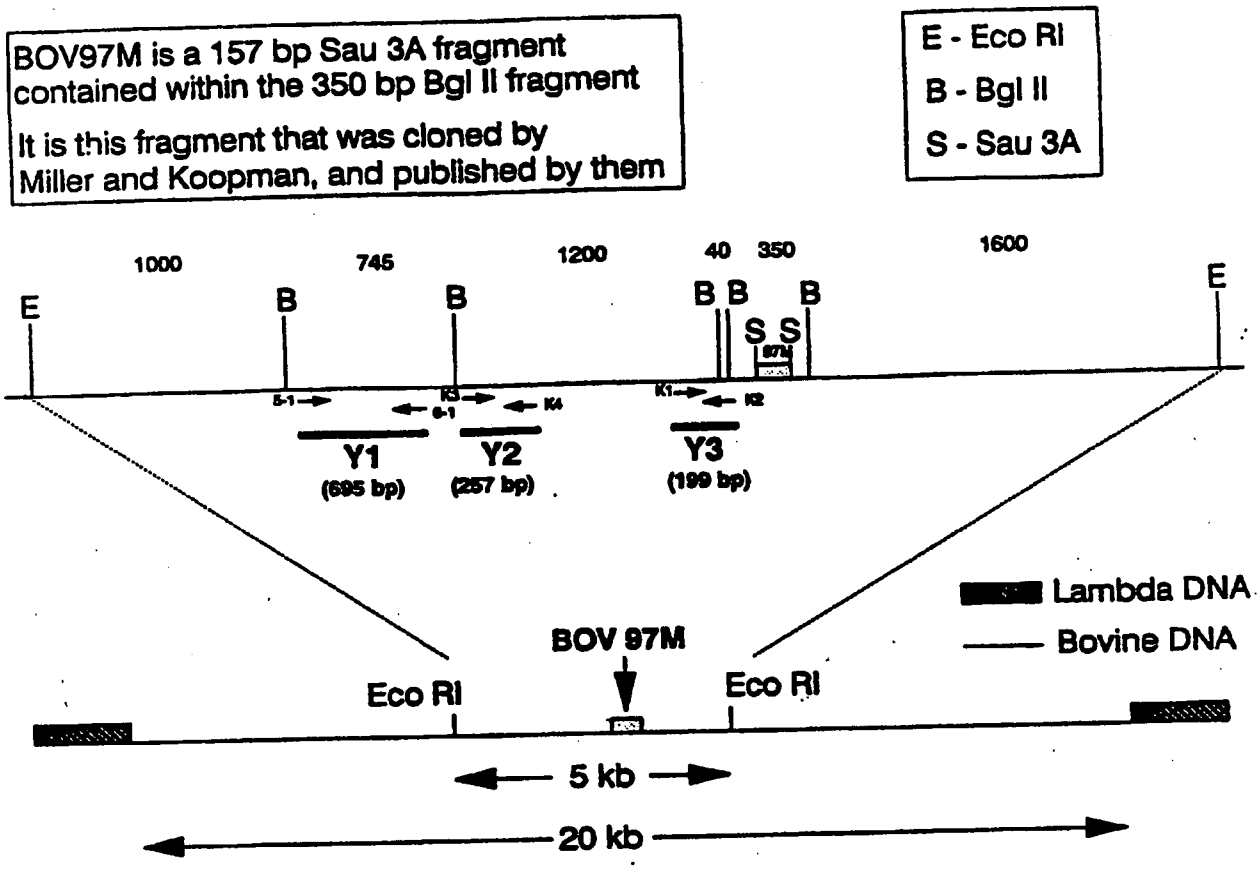
BOV97M is a 157 bp Sau 3A fragment contained within the 350 bp Bgl II fragment
It is this fragment that was cloned by Miller and Koopman, and published by them
E - Eco RI
B - Bgl II
S - Sau 3A
1000
745
1200
40
350
1600
E
B
B
B
B
S
S
E
Y1
(695 bp)
Y2
(257 bp)
Y3
(199 bp)
Lambda DNA
Bovine DNA
BOV 97M
Eco RI
Eco RI
5 kb
20 kb

FIGURE 3

*** INPUT INFORMATION ***

FILE: Y1.SEQ SEQUENCE: 745BP; 249 A; 134 C; 133 G;

*** SEQUENCE LIST *** (DOUBLE) 5-1 (25-mer)

```
          10         20         30         40         50         60
5' GATCTTGGGA GCAAGCAAAG TGTTTGGCCA CCTGAATAAT GTTGCCTAAA ATTTTATTTT
3' CTAGAACCCT CGTTCGTTTC ACAAACCGGT GGACTTATTA CAACGGATTT TAAAATAAAA
          70         80         90        100        110        120
   GACATATCCA CATACATACC CATGTGTGTA TGAGTTTGGG TATAAAAAAA TTACATTGAC
   CTGTATAGGT GTATGTATGG GTACACACAT ACTCAAACCC ATATTTTTTT AATGTAACTG
         130        140        150        160        170        180
   TTTTAATTCC CATTAGGTAT AACAACATGG AAAAATTAAA ACCCAAATGC AGAATGACTC
   AAAATTAAGG GTAATCCATA TTGTTGTACC TTTTTAATTT TGGGTTTACG TCTTACTGAG
         190        200        210        220        230        240
   AAGACTCTAC ACAGTTATTG AATATATGTA AGTGGAAATA CTTCAAAATA TGTTTTATTG
   TTCTGAGATG TGTCAATAAC TTATATACAT TCACCTTTAT GAAGTTTTAT ACAAAATAAC
         250        260        270        280        290        300
   GCTACATCTA ACATATAAGG ACTCTTTTAT ACCACTTCCC CTCTCTCCTT TTAATAAATA
   CGATGTAGAT TGTATATTCC TGAGAAAATA TGGTGAAGGG GAGAGAGGAA AATTATTTAT
         310        320        330        340        350        360
   CCTACCAATT TTCAAAGTTT GCCCAGGTTT ATGAAATACT CAAGGAATAA TAGGAAAAAT
   GGATGGTTAA AAGTTTCAAA CGGGTCCAAA TACTTTATGA GTTCCTTATT ATCCTTTTTA
         370        380        390        400        410        420
   ATATATATAT ATATATTATA TATATATATA GGAGAGGTAG GAACCAATCA TTACTCTGCT
   TATATATATA TATATAATAT ATATATATAT CCTCTCCATC CTTGGTTAGT AATGAGACGA
         430        440        450        460        470        480
   GGCCAATCCA TGGATGGAGG AGCCTGGTAG GCTGCAGTCC ATGGTGTCAC AAAATAGATC
   CCGGTTAGGT ACCTACCTCC TCGGACCATC CGACGTCAGG TACCACAGTG TTTTATCTAG
         490        500        510        520        530        540
   GGTACAACTG AGTGACTTCC CTTTCACTTT TCACTTTCAT GCATTGGAGA AGGACATAGC
   CCATGTTGAC TCACTGAAGG GAAAGTGAAA AGTGAAAGTA CGTAACCTCT TCCTGTATCG
         550        560        570        580        590        600
   AACCCACTCG AATGTTCTTG CCTGGAGAAT CCTGGGACAG ATGAGCCTGG TGGGCTGCCG
   TTGGGTGAGC TTACAAGAAC GGACCTCTTA GGACCCTGTC TACTCGGACC ACCCGACGGC
         610        620        630        640        650        660
   TCTGTGGGGT CACACAGAGT TGGACACGTC TGAAATGACA GCAGATATAC CTATTTATAT
   AGACACCCCA GTGTGTCTCA ACCTGTGCAG ACTTTACTGT CGTCTATATG GATAAATATA
         670        680        690        700        710        720
   GCATATTTAT GTATATAGAT ATACAAATAT CTATATAAAA TAGATATGTT TCCATTGCAG
   CGTATAAATA CATATATCTA TATGTTTATA GATATATTTT ATCTATACAA AGGTAACGTC
         730        740
   AAAATGTTGA TACCAACCTT AGATC 3'
   TTTTACAACT ATGGTTGGAA TCTAG 5'
```

6-1 (25-mer)

FIGURE 4 2113957

*** INPUT INFORMATION ***

FILE: Y2.SEQ SEQUENCE: 257BP; 92 A; 52 C; 48 G;

*** SEQUENCE LIST *** (DOUBLE)

K3 (26-mer)

```
          10        20        30        40        50        60
5' GATCTCAGAA TTATTTCCAA CACCATGCTC CAGGTCAACA GGAAATAGCC AAAGTTGTTG
3' CTAGAGTCTT AATAAAGGTT GTGGTACGAG GTCCAGTTGT CCTTTATCGG TTTCAACAAC
          70        80        90       100       110       120
   CTGACACAAA CAGTTCAAGA GCTGGGAAGC CTGTAGACCT CCTCATTTTC CAAGAGTAAT
   GACTGTGTTT GTCAAGTTCT CGACCCTTCG GACATCTGGA GGAGTAAAAG GTTCTCATTA
         130       140       150       160       170       180
   TTAATCAGAG AAGCTAGAAA TCCAGAAATT AAGGAAAATA ATCAAGCAAC AGAAGATAGT
   AATTAGTCTC TTCGATCTTT AGGTCTTTAA TTCCTTTTAT TAGTTCGTTG TCTTCTATCA
         190       200       210       220       230       240
   AATGATACTT TCACTTTTAA CTTTCATGCA TTGGAGAAGG AAATGGCAAC CCATTCCAGT
   TTACTATGAA AGTGAAAATT GAAAGTACGT AACCTCTTCC TTTACCGTTG GGTAAGGTCA
         250
   GTTCTTCCCT GGAGAAT 3'
   CAAGAAGGGA CCTCTTA 5'
```

K4 (25-mer)

FIGURE 5 2113957

*** INPUT INFORMATION ***

FILE: Y3.SEQ SEQUENCE: 199BP; 61 A; 39 C; 33 G;

*** SEQUENCE LIST *** (DOUBLE)

K1 (26-mer)

```
          10        20        30        40        50        60
5' ATAGATCTCA AAACTGAGTC CCTAGTAAGT GTATAATCAT ACAAAGAAGA TCTAACGGAG
3' TATCTAGAGT TTTGACTCAG GGATCATTCA CATATTAGTA TGTTTCTTCT AGATTGCCTC
          70        80        90       100       110       120
   GTTTCTGAAC CAGTTGATTC ATCCTTAGGA GTCCCATCTT GAATTTCTGA AGAAATATGT
   CAAAGACTTG GTCAACTAAG TAGGAATCCT CAGGGTAGAA CTTAAAGACT TCTTTATACA
         130       140       150       160       170       180
   GCCATCTCAA AGAAAGTCTT TCTTCAGATA TTTTCTTATT TGTCTGATTT ACACACTGCA
   CGGTAGAGTT TCTTTCAGAA AGAAGTCTAT AAAAGAATAA ACAGACTAAA TGTGTGACGT
         190
   ACTTCAAATG CTGCTTGGC 3'
   TGAAGTTTAC GACGAACCG 5'
```

K2 (26-mer)

FIGURE 6 2113957

*** INPUT INFORMATION ***

FILE: AUTO1.SEQ SEQUENCE: 174BP; 57 A; 37 C; 40 G;

*** SEQUENCE LIST *** (DOUBLE)

JC4 (26-mer)

```
          10        20        30        40        50        60
5' ACCAGACTTC TATAGTTGAA ACCTGTGCTG GATTCTGAGA GCAAGAAACC TCCACCAGCC
3' TGGTCTGAAG ATATCAACTT TGGACACGAC CTAAGACTCT CGTTCTTTGG AGGTGGTCGG
          70        80        90       100       110       120
   TGATAAAACT GCATGGGAGA GTTGGTTCCA GACTGAACAA TGTGAGCATG GAAAAGCCAT
   ACTATTTTGA CGTACCCTCT CAACCAAGGT CTGACTTGTT ACACTCGTAC CTTTTCGGTA
         130       140       150       160       170
   TCTTCTAATT AGATTGGCAG ACAGGAACAA CCTCTGCTAT GAGGTGAAAT ACAC 3'
   AGAAGATTAA TCTAACCGTC TGTCCTTGTT GGAGACGATA CTCCACTTTA TGTG 5'
```

JC7 (26-mer)

FIGURE 7 2113957

*** INPUT INFORMATION ***

FILE: AUTO2.SEQ SEQUENCE: 129BP; 32 A; 23 C; 28 G;

*** SEQUENCE LIST *** (DOUBLE)

JK1 (25-mer)

```
          10        20        30        40        50        60
5' GATCGAACTG ATGGTCCCTA TATTGCCAGG TGGATTCTTC ACTACTGGAC CACCAGGGAA
3' CTAGCTTGAC TACCAGGGAT ATAACGGTCC ACCTAAGAAG TGATGACCTG GTGGTCCCTT
          70        80        90       100       110       120
   GTCCCTGATG TGTTTTTTTG TTTTTTTTTT TTTTAAAGTG AAGGATAATG CAGCTAAAAT
   CAGGGACTAC ACAAAAAAAC AAAAAAAAAA AAAATTTCAC TTCCTATTAC GTCGATTTTA

   CAGCAGATC 3'
   GTCGTCTAG 5'
```

JK2 (25-mer)

FIGURE 8 2113957